Mitogen-Activated Protein Kinase Pathway-Dependent Tumor-Specific Survival Signaling in Melanoma Cells through Inactivation of the Proapoptotic Protein Bad

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ABSTRACT

Mitogen-activated protein kinase (MAPK) signaling regulates fundamental cellular functions including proliferation, differentiation, and survival. We have demonstrated previously that inhibiting MAPK signaling induces apoptosis in melanoma cells but not in normal melanocytes, suggesting that the MAPK pathway propagates essential survival signals in melanoma cells. Here, we report that the 90-kDa ribosomal S6 kinase (RSK), a downstream effector in the MAPK signaling cascade, phosphorylates and inactivates the Bcl-2 homology 3-only proapoptotic protein Bad, thereby mediating a MAPK-dependent tumor-specific survival signal in melanoma cells. The MAPK kinase (MEK)/extracellular signal-regulated kinase (ERK)/RSK MAPK signaling module is constitutively hyperactivated, and Bad is maintained in its inactive state by phosphorylation at Ser75 in a MEK/ERK/RSK-dependent manner in melanoma cells. In contrast, in normal melanocytes, Bad is highly phosphorylated at multiple residues (Ser75, Ser99, and Ser118) in a MAPK pathway-independent manner. Importantly, ectopic expression of a constitutively activated RSK mutant abrogates Bad activation and renders melanoma cells resistant to apoptosis induced by a MEK inhibitor. Furthermore, overexpressing alanine-substituted (S75A) Bad further sensitizes melanoma cells to MEK inhibitor-induced apoptosis. Our results suggest that the MAPK pathway mediates melanoma-specific survival signaling by differentially regulating RSK-mediated phosphorylation of the proapoptotic protein Bad and may present potentially selective therapeutic targets for the treatment of melanomas.

INTRODUCTION

MAPK pathways are highly conserved, ubiquitous signaling pathways regulating diverse cellular functions such as differentiation, proliferation, and survival. Although fundamental to maintaining cellular homeostasis, constitutive activation of Ras/Raf/MEK/ERK MAPK signaling is a hallmark of many human cancers, including breast, lung, and colorectal cancers as well as melanoma (1–5). Activating mutations in B-Raf have been documented in nearly 70% of human melanomas, with an additional 10–20% harboring activating Ras mutations (6, 7). Recent work by our laboratory and others identified both MEK and ERK as constitutively activated (CA) in human melanomas but not in normal melanocytes (8–10). Furthermore, we demonstrated that the MAPK pathway propagated survival signals specifically in melanoma cells and that inhibiting MAPK signaling in melanoma cells induced apoptosis and xenograft tumor regression (9). Collectively, these results suggest a causal role for MAPK signaling in melanomagenesis and survival. However, the downstream MAPK pathway effectors mediating melanoma-specific survival signals have yet to be identified.

The family of 90-kDa serine/threonine RSKs (p90RSK or MAPK-activated protein kinase 1) was among the first MAPK substrates identified (11). In humans, four separate genes encoding four isoforms (RSK1, 2, 3, and 4) were identified (11–13). Mutations in RSK2 are associated with Coffin-Lowry syndrome, an X-linked disorder characterized by mental retardation and skeletal abnormalities (12). RSKs have two distinct kinase domains and are activated by ERK and 3-phosphoinositide-dependent protein kinase 1 binding to and subsequent phosphorylation of RSK, as well as by autophosphorylation driven by the RSK COOH-terminal kinase domain (14–19). RSKs phosphorylate a variety of substrates via the NH2-terminal kinase domain, including the transcriptional factors cAMP-responsive element-binding protein, c-Fos, and Myt1 (20–22), and they regulate a diverse array of cellular functions such as gene transcription, protein synthesis, and cell cycle regulation (23). More recently, RSK was demonstrated to promote cell survival through phosphorylation and inactivation of the proapoptotic Bcl-2 family member Bad (22, 24). However, a role for RSK as an important downstream MAPK effector promoting survival in cancer cells has not been demonstrated.

The Bcl-2 family proteins are key regulators of apoptosis. Three subfamilies have been identified: (a) the prosurvival Bcl-2 (e.g., Bcl-2 and Bcl-XL) proteins; (b) the proapoptotic Bax (e.g., Bax and Bak) proteins; and (c) the BH3 domain-only (e.g., Bad, Bim, and Bid) proteins (25–27). Structurally, the BH3-only proteins are divergent from other Bcl-2 family members because they contain only one of the four conserved BH motifs (BH1–4). Normal cellular homeostasis requires the suppression of proapoptotic players by various mechanisms, including phosphorylation, intracellular localization, and heterodimerization with prosurvival Bcl-2 family proteins. Disruption of the balance between pro- and antiapoptotic Bcl-2 family members is suggested to be fundamental to the development of many diseases.

The BH3-only proapoptotic protein Bad is regulated through its phosphorylation and cytosolic sequestration. Dephosphorylated Bad promotes apoptosis by binding to either Bcl-2 or Bcl-XL and titrating them away from prosurvival Bax/Bak proteins. Unbound Bax oligomerizes and then disrupts mitochondrial integrity, causing cytochrome c release and initiating the caspase cascade (26, 28). However, phosphorylated Bad is bound and sequestered in the cytosol by the chaperone protein 14-3-3. Phosphorylation of one of at least three serine residues inactivates Bad by regulating interactions with either 14-3-3 or Bcl-2 family members: Ser75 (human designation; murine equivalent, Ser125), Ser99 (Ser136); or Ser118 (Ser155). Several survival kinases are implicated in the direct phosphorylation of Ser75, Ser99, and Ser118, including RSKs, Akt or p70S6K and PKA, respectively, indicating that Bad functions as an important convergence point in signal transduction pathways affecting cell survival. While RSK-mediated Ser75 phosphorylation or Akt- and/or p70S6K-driven Ser99 phosphorylation creates a consensus motif important for 14-3-3 binding and cytosolic sequestration (29–32), PKA-mediated Ser118 phos-
phorylation is proposed to induce conformational changes in the BH3 domain that disrupt interactions between Bad and prosurvival Bcl-2 family proteins (33–35). It is currently unclear which phosphorylation event(s) or which kinase(s) is important in regulating Bad activity in tumor cells.

In this study, we examined the importance of the downstream MAPK pathway effector proteins RSK and Bad in melanoma cell survival. Unlike in normal melanocytes, the MEK/ERK/RSK signaling module was constitutively hyperactivated and was required for melanoma cell survival. RSK-mediated Bad inactivation through Ser79 phosphorylation was necessary for maintaining cell survival in melanoma cells but not in melanocytes, in which an alternative MAPK-independent mechanism(s) may be responsible for survival. Our results identify MEK/ERK/RSK-mediated Bad inactivation as a critical mechanism promoting cell survival specifically in melanoma cells.

MATERIALS AND METHODS

Cell Culture, Inhibitors, and Antibodies. Human melanoma cell lines were obtained from the Developmental Therapeutics Program, National Cancer Institute/NIH and cultured in RPMI 1640 supplemented with 5% FBS, 2 mM l-glutamine, and 50 μg/ml gentamicin. For low-serum cultures, the medium was supplemented with 0.1% FBS plus 450 μg/ml BSA. NHEMs were obtained from Cascade Biologics (Portland, OR) and cultured in the recommended Medium 154 supplemented with 0.5% FBS, 0.2% bovine pituitary extract, 5 μg/ml bovine insulin, 5 μg/ml bovine transferrin, 3 ng/ml basic fibroblast growth factor, 0.18 μg/ml hydrocortisone, 3 μg/ml heparin, and 10 ng/ml phorbol 12-myristate 13-acetate. PD98059 (Cell Signaling Technology, Beverly, MA) was solubilized in DMSO, and cells were treated as described previously (9), unless otherwise stated. Cells were treated with either 2 or 5 μM PD184352 in DMSO (Upstate Biotechnology, Lake Placid, NY) for 72 h, which was sufficient to inhibit ERK1/2 activation completely in all melanoma cell lines tested (data not shown). Cells were treated once with 10 μM LY294002 (Sigma, St. Louis, MO) or 0.1 mM rapamycin (Calbiochem, San Diego, CA) solubilized in DMSO for 72 h before harvesting. Controls were treated with an equivalent volume of DMSO.

Antibodies to phospho-Bad Ser112 (7E11), phospho-RSK1/3 (T359/S363), caspase-9, PARP, phospho-ERK1/2, phospho-MEK1/2, and total MEK1/2 were obtained from Cell Signaling Technology. Antibodies to phospho-Bad Ser147, total Bad (H-168), total ERK1/2, and RSK3 (C20) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to RSK1 and RSK2 were obtained from Upstate Biotechnology. Antibody to phospho-Bad Ser99 was obtained from Biosource (Camarillo, CA). Antibody suitable for RSK1 immunoprecipitation was obtained from R&D Technologies (Minneapolis, MN). α-Tubulin antibody (DM1A) was obtained from Sigma.

Immunoprecipitation and Western Blotting. Lysates were prepared in SSB buffer (0.5% NP40, 0.1% Brij-35, 0.1% sodium deoxycholate, 1 mM EDTA, 7 mM KH2PO4, 3 mM KH2PO4, 5 mM EDTA, 10 mM MgCl2, 50 mM β-glycerol phosphate, 1 mM Na2VO4, 2 mM DTT, 5 μg/ml pepstatin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Total Bad antibodies conjugated to protein A beads (36) were used for immunoprecipitation. Total input protein for each immunoprecipitation reaction was 30 μg, and approximately 15 μg of Bad antibody was used per reaction. After overnight immunoprecipitation, beads were washed three times in SSB buffer and boiled in SDS sample buffer for subsequent Western blot analysis. Western blotting was performed as described previously (9).

Apoptosis Assays. Apoptosis was quantitated by either 4′,6-diamidino-2-phenylindole staining (9) or Annexin-V Alexa Fluor-568 (Molecular Probes, Inc., Eugene, OR) binding following the manufacturer’s recommended protocol. Flow cytometry was performed in a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA).

RSK1 Cloning and RT-PCR Analysis. Wt human RSK1 cDNA was cloned from normal melanocytes by RT-PCR into the TOPO Zero Blunt vector (Invitrogen Corp., Carlsbad, CA), and the sequence was verified with GenBank NM_002953.2. PCR-based site-directed mutagenesis was used to generate RSK1 mutants (Fig. 4A). CAI mutant contains an NH2-terminal myristoylation signal (MGSSKKSPK) and the Y702A substitution (24), as well as phospho-mimic substitutions EED99 and EDD99 in the regulatory linker region (17). CAII lacks the first 43 NH2-terminal residues but contains Y702A and the phospho-mimic substitutions as described in CAI (17, 37, 38). A KD mutant harbors S221A and T573A substitutions in catalytic domains and K94A and K447A substitutions in ATP-binding domains (16, 17, 24, 37, 38).

All RSK1 and control LacZ constructs were subcloned into pShuttle-cytomegalovirus IRES-GFP (Stratagene, La Jolla, CA) for transfection experiments. RT-PCR analysis of human RSK isoforms was performed using the following primers: (a) RSK1 5′-RT (5′-GAGGCAACAGGCTTCCGG-3′) and RSK1 3′-RT (5′-GCTTGAGCGAGAAGGTCC-3′); (b) RSK2 5′-RT (5′-AGC-GGGTGGACCTGGGCCC-3′) and RSK2 3′-RT (5′-GACCCCGGGGCTCT-GAAAGA-3′); and (c) RSK3 5′-RT (5′-TGCTGTCCTTGTGCTGACAG-3′) and RSK3 3′-RT (5′-ACCATGATCCAAATATC-3′).

Transfections and CD4 Selection. Transfections were performed using LipofectAMINE 2000 Reagent (Invitrogen Corp.) following the manufacturer’s recommended protocol, with minor modifications. Cells were transfected overnight and washed in cold PBS, and growth media were added. For CD4 cotransfections, a 2:1 ratio DNA dilution of the experimental vector to the PMACS 4-IREs.II CD4-encoding vector (Miltenyi Biotec, Auburn, CA) was used. Melanoma cells cotransfected with CD4-expressing vectors were purified using magnetic beads coated with anti-CD4 antibodies using the manufacturer’s recommended protocol (Dynabech, Tech, Lake Success, NY). For thes treatments, selected cells were plated overnight in growth media and treated with PD98059 as described above.

Retroviral Vectors and VSG-G Pseudotyped Virus Production. An IRES-GFP fragment was subcloned into the Moloney murine leukemia virus-based retroviral vector pLPCX (Becton Dickinson, Franklin Lakes, NJ). All RSK1 and control LacZ constructs were subcloned into the resulting vector such that GFP expression is dependent on RSK or LacZ expression. Each vector was transfected into the helper line 293-GFP (a kind gift from Dr. Cindy Miranda, Van Andel Research Institute) to produce VSV-G pseudotyped replication-defective viral stocks. Viral titers were determined on HEK293 cells to equal multiplicity of infection when infecting melanoma cell lines.

RSK1 in Vitro Kinase Assays. RSK in vitro kinase assays were performed as described previously (24) with minor modifications. RSK1 immunoprecipitations were performed overnight with rotation at 4°C using 300 μg of total input protein. Kinase assays were performed using 0.25 μg of GST-mouse Bad fusion protein as a substrate.

Stable Bad Transfectants. Wt human Bad or alanine-substituted Bad (S75A) cDNA was cloned into pDEST26 vector through Gateway cloning technology (Invitrogen Corp.). M1-4-MEL melanoma cells were transfected with human Bad WT, human Bad S75A, or empty pDEST26 vector using LipofectAMINE 2000 Reagent (Invitrogen Corp.), and pools of stable transfectants were generated by selecting for G418 resistance.

RESULTS

The MEK/ERK/RSK Signaling Module Is Constitutively Hyperactivated, and Proapoptotic Bad Is Differentially Phosphorylated in Melanoma Cells. We demonstrated previously that MEK inhibitors, including anthrax lethal toxin, induced apoptosis in a panel of human melanoma cell lines but induced G1 arrest in NHEMs (9). Consistent with the previous results, an additional small molecule MEK inhibitor, PD184352 (39), induced apoptosis in all seven melanoma cell lines tested because activated ERK1/2 were still detected in melanoma cells.

We first examined MEK1/2 and ERK1/2 expression and activation in a cohort of melanoma cell lines and in normal melanocytes (Fig. 1A). As described previously (9), although comparable levels of MEK1/2 and ERK1/2 expression were detected in melanoma cells and melanocytes, activation of both MEK1/2 and ERK1/2 was relatively higher in melanoma cells. Furthermore, consistent with previous findings (10), ERK1/2 activation was constitutive in all melanoma cell lines tested because activated ERK1/2 were still detected in melanoma cells cultured in low (0.1%) serum (Fig. 1C). We next evaluated the expression and activation of RSKs, which were previously implicated

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in MAPK-mediated cell survival (18, 21, 22, 24, 38, 40, 41). Preliminary RT-PCR analysis using isoform-specific primers followed by Western blotting showed that three isoforms (RSK1, 2, and 3) were detected in the melanocytes and melanoma cells examined; however, among the RSKs analyzed, RSK1 was overexpressed in some and hyperactivated in all melanoma cell lines tested (Fig. 1B). Thus, these results demonstrate that the MEK1/2-ERK1/2-RSK1 signaling module is constitutively hyperactivated in melanoma cell lines relative to cultured normal melanocytes. There was no additional ERK1/2 or RSK1 activation induced with the higher (5%) serum content in melanocytes (Fig. 1D), indicating that the differential MEK1/2-ERK1/2-RSK1 activation in melanoma cells versus melanocytes was not attributable to the differences in serum content in their respective culture media (Fig. 1, C and D).

The proapoptotic function of the Bcl-2 family protein Bad is regulated by its phosphorylation state and is a crucial effector of many survival kinases, including RSKs (22, 24, 42–44). We next determined the phosphorylation state of Bad in melanocytes and melanoma cell lines. Bad was highly phosphorylated at Ser75 in both melanoma cells and melanocytes (Fig. 1E). However, whereas Bad was phosphorylated at Ser99 and Ser118 in melanocytes, these phosphorylations were minimally detected in melanoma cell lines, with the exception of UACC-62 (Fig. 1E). Similar levels of Bad protein expression were found in melanocytes and melanoma cells (Fig. 1E).

**Bad Phosphorylation at Ser75 Is Dependent on ERK/RSK Activation in Melanoma Cells but not in Normal Melanocytes.** To determine whether RSK activation and Bad phosphorylation are dependent on MAPK signaling, we treated normal melanocytes and melanoma cell lines with MEK inhibitors, either PD98059 or PD184352. In melanoma cells, ERK1/2 and RSK activation as well as ERK1/2-RSK1 activation in melanoma cells versus melanocytes was unaffected by MEK inhibition in all melanoma cells except UACC-62 cells (Fig. 2). Similar levels of Bad protein expression were found in melanocytes and melanoma cells (Fig. 1E).

Unpublished observations.
sensitive, in part, to MEK inhibition (Fig. 2B), suggesting cross-talk between the MAPK pathway and alternative/additional survival pathways. In fact, significantly enhanced activation of additional survival kinases including Akt, p70S6K, and 3-phosphoinositide-dependent protein kinase 1 was detected in UACC-62 cells when compared with other melanoma cell lines examined (data not shown).

**Loss of Bad Ser\(^{75}\) Phosphorylation Correlates with the Onset of Apoptosis in Melanoma Cells.** To determine the kinetics by which MEK inhibition induces apoptosis, M14-MEL and MALME-3M melanoma cells were treated with PD98059 and monitored at the indicated time points for cell death, as well as for (in)activation of ERK/RSK and downstream apoptotic signaling cascades. Significant cell death was observed in M14-MEL cells within 36 h of treatment (Fig. 3A). PD98059 treatment had slightly delayed and less dramatic apoptotic effects on MALME-3M cells (Fig. 3C). Within 4 h of treatment, both ERK1/2 and RSK activation were inhibited in M14-MEL cells (Fig. 3B). Bad Ser\(^{75}\) phosphorylation was diminished within 8 h of treatment and was not detected after 36 h. The gradual loss of Bad Ser\(^{75}\) phosphorylation correlated with the onset of caspase-mediated death signaling (25–27) in M14-MEL cells, as evidenced by activation of caspase-9 and subsequent cleavage of a caspase-3 substrate, PARP (Fig. 3B). Similarly, RSK activation in MALME-3M melanoma cells was inhibited within 4 h of treatment; the loss of Bad Ser\(^{75}\) phosphorylation was evident only after 36–48 h of treatment and was associated with increased apoptosis (Fig. 3, C and D). These results suggest that loss of Bad Ser\(^{75}\) phosphorylation (i.e., activation) is consistent with the initiation of MEK inhibition-induced apoptotic response in melanoma cells.

**Ectopic Expression of a CA RSK1 Protects Melanoma Cells from Apoptosis Induced by MEK Inhibitor.** To evaluate the specific contributions of RSK to melanoma cell survival through Bad phosphorylation, we next examined the effects of ectopically expressing Wt, CA, or KD RSK1 constructs (Fig. 4A) on apoptosis induced by MEK inhibition. A Lac\(^{-}\)expression construct served as a negative control in these experiments. Expression and catalytic activities of RSK1 constructs were confirmed by transient expression in HEK293 cells (Fig. 4B) and by in vitro kinase assays using recombinant GST-mouse Bad fusion protein as a substrate. Although overexpressing Lac\(^{-}\)did not enhance Bad Ser\(^{112}\) (Ser\(^{75}\) equivalent in human Bad) phosphorylation beyond background (GST-Bad alone), overexpressing Wt RSK1 induced significant Bad Ser\(^{112}\) phosphorylation. Expressing CAI or CAII RSK1 yielded further enhanced Bad Ser\(^{112}\) phosphorylation when compared with Wt, although KD RSK1 was unable to phosphorylate Bad Ser\(^{112}\) beyond Lac\(^{-}\} levels. Comparable RSK1 expression was observed in all transfectants (Fig. 4B, bottom panel). We then confirmed the activities of RSK1 constructs toward endogenous Bad in M14-MEL cells. Because of extremely low transfection efficiencies observed in melanoma cells (data not shown), we cotransfected RSK1 constructs with a vector expressing a CD4 cell surface antigen, and the resulting transfectants were enriched using magnetic beads coated with anti-CD4 antibody. The enriched transfectants were then treated with or without PD98059, and the phosphorylation of Bad Ser\(^{75}\) was evaluated. In this transient transfection system, CAI and CAII RSK1-expressing melanoma cells sustained Bad Ser\(^{75}\) phosphorylation in the presence of PD98059, whereas in Lac\(^{-}\}-expressing cells, Bad Ser\(^{75}\) phosphorylation was blocked (Fig. 4C). Although overexpressed Wt RSK1 induced Bad Ser\(^{112}\) phosphorylation in the in vitro kinase assay (Fig. 4B), Bad Ser\(^{75}\) phosphorylation was not detected in M14-MEL cells expressing Wt RSK1 and treated with PD98059 (Fig. 4C). This discrepancy may be attributed to experimental difficulties such as low transfection efficiencies and uneven loading. Overexpression of KD RSK1 did not affect Bad Ser\(^{75}\) phosphorylation in untreated cells (Fig. 4C).

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Fig. 2. MEK inhibitors block RSK activation and Bad Ser\(^{75}\) phosphorylation in melanoma cells. A, NHEM, MALME-3M, M14-MEL, and UACC-62 cell lines were treated with either DMSO (−) or 20 μM MEK inhibitor PD98059 (+) for 72 h, and whole cell lysates were prepared. Western blotting was performed using the indicated phospho-specific antibodies to ERK1/2 or RSK1, and the blots were reprobed with their respective total protein antibodies. A representative α-tubulin reprobed blot is shown in the bottom panel as a loading control. B, using the same set of lysates as described in A, immunoprecipitation with an anti-total Bad antibody was performed, and the immunoprecipitates were blotted with phospho-specific antibodies against Bad Ser\(^{75}\), Ser\(^{99}\), or Ser\(^{118}\). Blots were reprobed with an antibody recognizing total Bad protein. C, NHEM, MALME-3M, and M14-MEL cell lines were treated with either DMSO (−) or 2 μM MEK inhibitor PD184352 (+) for 72 h and analyzed as described in A and B.
To evaluate the effects of expressing RSK1 constructs on melanoma cell survival, we used a VSV-G envelope protein pseudotyped Moloney murine leukemia virus-based retroviral vector system with an IRES-GFP expression cassette (45). This system allowed for enhanced gene transfer efficiency through infection, and infected cells were identified by GFP coexpression. RSK1 expression was confirmed in M14-MEL cells, with each infection yielding comparable levels of RSK1 expression (data not shown). Infected M14-MEL and MALME-3M cells were treated with PD98059 and analyzed by flow cytometry for apoptosis. Overexpression of Wt, CAI, or CAII RSK1 partially protected both M14-MEL and MALME-3M cells from apoptosis induced by PD98059, whereas KD RSK1 was ineffective (Fig. 4). Wt RSK1 expression reduced PD98059-induced apoptosis by ~50%, whereas either CAI or CAII RSK1 expression reduced apoptosis by >75% (Fig. 4F). Overexpressing KD RSK1 did not affect overall PD98059-induced apoptosis (Fig. 4F). These results, therefore, implicate RSK in mediating a melanoma-specific survival signal.

**Bad(S75A) Mutant Further Sensitizes Melanoma Cells to MEK Inhibitor-Induced Apoptosis.** If Bad Ser^75^ phosphorylation functions in melanoma cell survival, alanine substitution of Ser^75^ would constitutively activate Bad, enhancing the apoptotic response to MEK inhibition. To evaluate whether Bad(S75A) mutant expression sensitizes melanoma cells to apoptosis induced by a MEK inhibitor, we established stable pools of M14-MEL cells expressing either Wt human Bad or Bad(S75A). We found severalfold greater expression of Wt Bad than Bad(S75A) in these stable lines (Fig. 5A). Expression of Bad(S75A) significantly sensitized M14-MEL cells to PD98059-induced apoptosis compared with control-transfected cells (Fig. 5B). Sensitization to PD98059 was dose dependent because cells expressing Bad(S75A) were approximately twice as sensitive as control-transfected cells to apoptosis induced by 20 μM PD98059, whereas only moderate sensitization was observed at 5 μM PD98059 (Fig. 5B). Although a trend was noted, sensitization by Wt Bad was not statistically significant because of comparatively large SDs (Fig. 5B). Moreover, Wt Bad expression was severalfold greater than that of Bad(S75A) (Fig. 5A); hence, with protein levels normalized to apoptosis, Bad(S75A) was significantly more potent than Wt Bad in enhancing apoptotic sensitivity of melanoma cells to PD98059 (Fig. 5B). Taken together, these results indicate that MAPK/RSK-dependent Bad inactivation by Ser^75^ phosphorylation is an important mediator of melanoma survival signaling. However, the finding that Bad(S75A) expression is tolerated by melanoma cells (Fig. 5A) suggests that inactivation of Bad alone may not be sufficient for melanoma cell survival. It is conceivable that additional survival signaling molecules are required to fully confer melanoma cell survival. Furthermore, we cannot rule out the possibility that these stable transfectants may have independently developed an adaptive mechanism to overcome the selective pressure of Bad-induced apoptosis.

**DISCUSSION**

Data presented in this study reveal that the MAPK pathway functions in a melanoma cell-specific way to negatively regulate the activity of proapoptotic Bcl-2 family protein Bad. Unlike in normal human melanocytes, Bad is inactivated through hyperactivation of the MEK/ERK/RSK signaling module, and inhibiting this pathway effectively induces apoptotic cell death. Taken together, our findings indicate that constitutive activation of MEK/ERK/RSK signaling and

![Image](image-url)
sustained Bad inactivation provide a tumor-specific survival mechanism for melanoma cells.

Constitutive activation of MAPK signaling is a hallmark of many human cancers, including breast and colon cancers and melanoma (1, 3, 39, 46). Constitutive activation of both MEK and MAPK was demonstrated in melanoma but not in benign nevi (2, 8). Expression of CA MEK in immortalized melanocytes promotes tumor formation in nude mice (8). Recently, a high incidence of activating mutations in B-Raf, an upstream activator of the MEK/ERK signaling, has been found in melanoma cell lines, atypical nevi, and primary tumors (4, 6, 10, 47), indicating the potential importance of B-Raf and downstream MAPK signaling to neoplastic melanoma transformation.

RSKs are implicated in the regulation of a wide variety of cellular processes, including gene expression, proliferation, and survival. In humans, mutations in the RSK2 isoform are associated with Coffin-Lowry syndrome, a disease characterized by mental retardation and skeletal abnormalities (12). However, the role of RSKs in human cancers has not been established. Our results demonstrate that RSK is both overexpressed and hyperactivated in melanoma cell lines relative to normal melanocytes (Fig. 1B). To our knowledge, this is the first demonstration of enhanced RSK expression and activation in tumor cells. Additional studies may help elucidate whether RSK expressed in melanoma cells harbors activating mutations like those found in Ras and B-Raf, or whether its activation is directly attributable to activating mutations in upstream Ras or B-Raf.

RSK specifically phosphorylates Bad at Ser 75 in a MAPK-dependent manner (22, 24, 42–44), facilitating its inactivation through binding to 14-3-3 and sequestration from heterodimerizing with mitochondria.

Fig. 4. Ectopic expression of CA RSK1 mutant protects melanoma cells from PD98059-mediated apoptosis. A, schematic of Wt, CAI, CAII, and KD RSK1 constructs. Important residues for RSK activity and corresponding mutations are indicated, and the COOH-terminal and NH2-terminal kinase domains are represented by gray boxes. An NH2-terminal myristoylation sequence is indicated (black box) in the CAI construct. B, expression and catalytic activities of the RSK1 constructs were characterized by transient transfection assays in HEK293 cells with a LacZ expression construct as a negative control. RSK1 immunoprecipitates were prepared and subjected to an in vitro kinase assay as described in “Materials and Methods” using recombinant GST-mouse Bad as a substrate. Bad Ser112 (equivalent to human Bad Ser75) phosphorylation was analyzed by Western blotting using an antibody specific to phospho-Bad Ser112. GST-Bad alone is loaded as a negative control, and anti-GST blots are shown as substrate loading controls for the kinase assay. RSK1 expression was confirmed by direct Western blotting (bottom panels). C, transfectants transiently expressing RSK1 constructs were enriched based on coexpression of cell surface CD4 antigen and selection with magnetic beads coated with anti-CD4 antibody. Lysates were prepared from the transfectants treated with 20 μM PD98059 for 48 h. Bad Ser75 phosphorylation and total Bad protein levels were assayed by immunoprecipitation/Western blot analysis. The altered mobility of the KD RSK1 band was attributable to a gel anomaly. D, VSV-G pseudotyped retroviral vectors containing the RSK1 constructs (A) linked to IRES-GFP were used to infect M14-MEL melanoma cells. The LacZ construct-containing virus was used as a control. Seventy-two h after infection, the infected cells were treated in triplicate with DMSO or 20 μM PD98059 for 72 h and analyzed by flow cytometry for apoptosis in the gated (GFP-positive) population of cells. Data are expressed as average ± SD. F, M14-MEL cells infected with the LacZ or RSK1 viruses were enriched by selecting for puromycin resistance. RSK1 expression in the enriched populations was confirmed by Western blotting (top panel). Apoptotic response to PD98059 of the enriched populations was analyzed as described in D. Significance of the differences in apoptotic sensitivity of each PD98059-treated sample compared with the corresponding LacZ control (D–F) was analyzed with a two-tailed Student’s t test (equal variances): *, P < 0.004; **, P > 0.375; and ***, P > 0.093.
Although a plethora of data exists documenting the proapoptotic role of Bad, only recently have the consequences of Bad Ser75/Ser99/Ser118 phosphorylation been examined in vivo. Using a knock-in approach, Datta et al. (51) generated a Bad S3A mouse in which all three regulatory serines were substituted with alanines, thereby preventing phosphorylation by survival kinases. Although this mouse was viable, suggesting that elements of survival signaling are still functional in the presence of Bad S3A, subsequent analysis documented defects in developmentally regulated apoptosis in certain cell types from the nervous and immune systems. Hence, although insufficient to induce general cell death during development, Bad dephosphorylation is necessary to regulate apoptosis in certain tissues, potentially through collaboration with additional proapoptotic signaling molecules. To this end, expressing CA FOXO3A, a Forkhead transcriptional factor inducing several genes promoting cell death, dramatically enhanced apoptotic response in cerebellar granule neurons derived from Bad S3A mice, suggesting that Bad S3A sensitizes cells to the effects of certain death-inducing stimuli in vivo. Furthermore, Bad S3A sensitization was demonstrated to alter the threshold at which mitochondria release cytochrome c in response to apoptotic stimuli. The notion that Bad Ser75/Ser99/Ser118 dephosphorylation is necessary yet not sufficient to induce apoptosis in certain cell types in vivo may suggest a critical, but not exclusive, role for Bad phosphorylation in promoting melanoma cell survival in vitro (Fig. 5).

Our results suggest that the MAPK pathway mediates melanoma-specific survival signaling by differentially regulating RSK-mediated phosphorylation of the proapoptotic protein Bad, but not in normal melanocytes. Therefore, interfering with the MEK/ERK/RSK signaling and selectively activating proapoptotic mediators may represent a potential therapeutic strategy in the treatment of melanoma.

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